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## Oxidative Stress Does Not Predispose Neuronal Cells to Changes in G Protein Coupled (Opioid) Receptor Gene Expression in Cortical B50 Neurons in Culture

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**ABSTRACT** Oxidative stress adversely affects neuronal cells in which they may die when oxygen supply is reduced or eliminated and opioid receptor agonists elicit several central nervous system effects. The aim of this study was to evaluate the effect of oxidative stress on opioid receptor gene expression in cortical B50 cells. The cells were cultured in normoxia, hypoxia and treated with opioid agonists; DAMGO ( $\mu$ ), DSLET ( $\delta$ ) and ICI—199,441 ( $\kappa$ ) for 48 hours after 48 hours of initial culture at dose of 10 $\mu$ M, 50 $\mu$ M and 100 $\mu$ M. The level of mu opioid receptor mRNA was assessed using RT-PCR. The results show that oxidative stress induced changes in B50 cells in hypoxia while mu opioid mRNA levels showed no change. The results show that B50 cells are susceptible to damage by oxidative stress and opioid agonist treatments showed no change in the level of mu opioid receptor gene expression in B50 cells.

### INTRODUCTION

Oxygen deprivation can lead to oxidative stress which has been implicated in nerve cell death that occurs in a variety of neurodegenerative disorders like dementias, multiple sclerosis, Alzheimer's disease and Parkinson's disease (Benzi et al. 1994; Maher 2001; Prentice et al. 2011). Oxidative stress leads to metabolic cellular processes in which oxidative species such as super oxide radical anions, hydrogen peroxide and lipid peroxides are generated intracellularly (Scandalios 1997; Chen and Buck 2000; Lee et al. 2012). These reactive species, if not eliminated, may damage DNA, proteins or membrane lipids and cause oxidative cell death. Endogenous antioxidative enzymes as well as antioxidants are required for cells to survive (Semenza 2005), while exogenous antioxidants have been shown to effectively prevent oxidative cell death in cultured cells (Busciglio and Yankner 1995; Nakao et al. 1996; Du et al. 2012).

Hypoxic stress results in a rapid and sustained inhibition of protein synthesis that is partially mediated by eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) phosphorylation by the phospho-endoplasmic reticulum kinase (PERK) (Blais et al. 2004). Severe hypoxia has been shown to induce apoptotic cell death in developing brain neurons whereas mild hypoxia has been demonstrated to stimulate neurogenesis (Bossenmeyer-Pourie et al. 2002; Yeh et al. 2008). Hypoxia threatens brain function throughout the entire life span and although the physiological consequences of brain hypoxia are well documented, the molecular mechanisms involved in these processes are still not well understood (Rossler et al. 2001; Zhu et al. 2005; Semenza 2006, Semenza 2007). It has been shown that hypoxia may have severe detrimental effects on most cells and especially on neuronal cells. The responses of cells to hypoxia may involve the induction of specific gene expression which may help to suppress the effects of hypoxia in cells (Yun et al. 1997; Kim et al. 2012; Lee et al. 2012).

Gibson and Huang (2002) used the oxidative processes in the brain as a biomarker of Alzheimer's disease and showed that diminished metabolism and excessive oxidative stress occur in brains of patients with Alzheimer's disease. It

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has been observed that oxidative stress can cause neurodegeneration associated with enhanced susceptibility to apoptosis due to the activation of pro-apoptotic genes (Gibson and Huang 2002; Du et al. 2012). Hypoxia-induced oxidative stress can cause neurite retraction leading to neurodegeneration and neuronal loss similar to that found in Alzheimer's disease (Banasiak et al. 2000; de la Monte et al. 2000; Yeh et al. 2008; Prentice et al. 2011; López-Hernández et al. 2012).

Opioid receptors, found in the central nervous system are classified as mu ( $\mu$ ), kappa ( $\kappa$ ), delta ( $\delta$ ) and sigma ( $\sigma$ ) opioid receptors. These receptors are not uniformly distributed in the CNS with the highest concentration in the cerebral cortex (Raynor et al. 1996; Chaturvedi et al. 2000). The mu receptor has been shown to be high in areas of pain perception and in the medulla, especially in the area for respiration (Hasbi et al. 2000; Fichna et al. 2006; Li et al. 2012).

The opioid receptors (mu, delta, and kappa) belong to the large family of GPCRs and have diverse and important physiological roles (Piestrzeniewicz et al. 2006). Laugwitz et al (1993) have shown that activated delta opioid receptors are coupled to  $G_{i1}$  while activated mu opioid receptors are coupled to  $G_{i3}$  in neuroblastoma cells. Mu opioid receptors have been shown to be activated by mu receptor agonists and are coupled through the  $G_{\alpha_{i1}}$  and  $G_{\alpha_{iA}}$  in human embryonic kidney cells (Saidak et al. 2006). Tso and Wong (2000) have shown that both mu and kappa opioid receptors are coupled via both  $G_i$  and  $G_o$  in HEK 239 cells. The stimulation of opioid receptors triggers analgesic effects and affects the function of the nervous system, gastrointestinal tract and other body systems (Piestrzeniewicz et al. 2006; Wang et al. 2007; Vermehren-Schmaedick et al. 2012).

### Objective of the Study

The aim and the objectives of the present work was to investigate the expression of opioid (mu) receptor gene in B50 neuronal cells, to investigate the effect of oxidative stress using hypoxia on the expression of mu opioid receptor gene on B50 cells and to study the effect of mu opioid receptor agonist treatment on opioid (mu) receptor gene in cultured B50 cells using semi quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR).

## MATERIAL AND METHODS

### Neuronal Culture

One group of B50 neuronal cells were cultured and maintained in a normoxic incubator (21%  $O_2$ ; 5%  $CO_2$ ) as control group and another batch of cells cultured under hypoxia (5%  $O_2$ ; 5%  $CO_2$ ) as experimental group. Cells were cultured in 12-well culture plates for 48 hours and were treated with opioid agonists to determine their effects on hypoxia-induced changes. Three opioid agonists {DAMGO ( $\mu$ ), DSLET ( $\delta$ ) and ICI—199,441( $\kappa$ )}, and antagonists were administered to the cells as treatment against hypoxia for 48 hours for a total of 96 hours at a concentration of 10 $\mu$ M, 50 $\mu$ M and 100 $\mu$ M. The total cellular Ribonucleic acid (RNA) was extracted from the cultured B50 neuronal cells using the TRIzol reagent method (Invitrogen No 15596-026).

### Method

The B50 cells in the different groups were grown and lysed in culture plates by adding 0.5ml of TRIzol reagent to each well. The cells were homogenized and incubated for 5mins at room temperature. The homogenates were transferred to micro-centrifuge tubes, 0.1ml of chloroform added, the cap secured and the tubes shaken vigorously by hand for 15 seconds. The cellular mixture was incubated at room temperature for 3 minutes. The mixture was then centrifuged at 12,000 x g for 15 minutes at room temperature. Following centrifugation, the mixture, separated into 3-layers namely a lower phenol-chloroform phase (Red), a middle interphase (Cloudy) and an upper aqueous colourless phase. The RNA was present at the upper aqueous phase and formed about 60% of the total volume of the mixture and was transferred to a fresh micro-centrifuge tube. The aqueous phase was mixed with 0.25ml of isopropyl alcohol and incubated for 10 minutes at room temperature. The mixture was then centrifuged at 12,000 x g for 10 minutes at room temperature. At this point the RNA precipitated and formed a gel-like pellet.

The supernatant was removed and the remaining RNA pellet was washed once with 0.5ml of 75% ethanol and mixed by vortexing. The mixture was centrifuged at 7,500 x g for 5

minutes at room temperature and the ethanol was decanted. The RNA was then air dried for 10 minutes, dissolved in 100% deionized formamide and stored at -70°C to be used in RT-PCR analysis.

#### **Semi-quantitative One step RT-PCR Analysis**

The semi-quantitative one step reverse transcriptase polymerase chain reaction (RT-PCR) was used to study the gene expression of mu opioid receptors. Mu opioid receptor (MOR) was selected for the semi-quantitative RT-PCR analysis because opioid receptor subtypes have been shown to have 68% sequence homology between them and the differences between them was proposed largely, on the basis of radioligand binding studies and as such there is little or no evidence for the presence of the different genes encoding the opioid receptor subtypes (Corbett et al. 2006). In some cases receptor heterodimerisation of opioid receptors has been proposed as a possible explanation for the different opioid receptor subtypes (Milligan 2004).

The extracted total RNA, Superscript III RT/Platinum Taq Mix, Reaction Mix, 5mM Magnesium Sulphate, GeneAMP PCR System thermal cycler were used according to the manufacturer's instruction. Mu opioid receptor (MOR) sense primers, 5'-GGA ACA TGG CCC TTC GGA ACC ATC-3' (574-597) and antisense 5'-TAC CAG GTT GGG TGG GAG AAC GTG-3' (863-840), were selected from Silbert et al. (2003), where they were used to study the effect of MOR expression and opioid treatment in myelinated and unmyelinated neurons. Alpha actin primer Sense 5'-GAT CAC CAT CGG GAA TGA ACG C-3' (389bp) and Antisense 5'-CTT AGA AGC ATT TGC GGT GGA C-3', selected from Park et al. (1997), where they were used as an internal control for cytoskeletal study in pericytes.

Programming of the thermal cycler was done as follows: cDNA synthesis 1 cycle at 55°C for 30 minutes, Denaturation 1 cycle at 94°C for 2 minutes, PCR amplification 40 cycles at 94°C for 15 seconds (Denature), 60°C for 30 seconds (Anneal), 68°C for 60 seconds (Extend), Final extension, 1 cycle at 68°C for 5 minutes.

The master mix was prepared on ice using 0.2ml nuclease free, thin walled PCR tubes. Each PCR tube contained the following: 2 x

Reaction mix (dNTPs: 200μM; MgSO<sub>4</sub>:1.6mM) 25μl, Template RNA (200ng/μl) 1μl, Sense Primer (0.2μM), 1μl, Antisense Primer (0.2μM) 1μl, SuperScript III RT/ Platinum Taq mix (5mM) 2μl, Autoclaved distilled water; 20μl was added to make up total volume to 50μl.

These were mixed together gently and all the components were allowed to settle at the bottom of the amplification tube. The tubes were then centrifuged briefly by pulse centrifugation, overlaid with one drop of mineral oil and placed in a preheated thermal cycler (GeneAMP PCR System) as programmed above. One tube was used as blank and contained only master mix and water.

#### **Analysis of the RT-PCR Products**

The analysis of the PCR products was carried out using the following method. Agarose gel (2%) was made by dissolving 2.5g agarose in 112.5ml of distilled water which was subsequently micro-waved in a conical flask for 5 minutes. 12.5ml buffer solution of Tris/Borate/EDTA (TBE) was added and 5μl of ethidium bromide added for staining the mRNA and mixed thoroughly. The mixture was poured into the gel tray with combs in place and air bubbles pushed to the sides with a pipette. The gel was allowed one hour to set. 5μl of Blue loading buffer (Sigma, G7654), which contain bromophenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), sucrose (40% w/v), was added to each of the samples and centrifuged briefly to mix and settle.

A 10μl deoxyribonucleic acid (DNA) ladder (Promega G2101) and 5μl Blue loading buffer was used to make-up the DNA marker, and one litre of gel buffer was made using the TBE in a 1:10 dilution. The gel was placed in an electrophoresis tank with wells at the negative electrode such that RNA will move towards the positive electrode. The gel was then surrounded with gel buffer and the wells completely covered by the buffer. The DNA marker (5μl), was added to the gel on position one, followed by adding a 10μl blank sample to the next position, and continuing to add 10μl of each sample to the remaining wells. The electrophoresis tank was connected to a power supply set at 125V for one hour. Photographs of the gels were taken and scanned using the digital densitometer to evaluate and semi-quantify the mRNA of the

receptors, and then compared between the different groups.

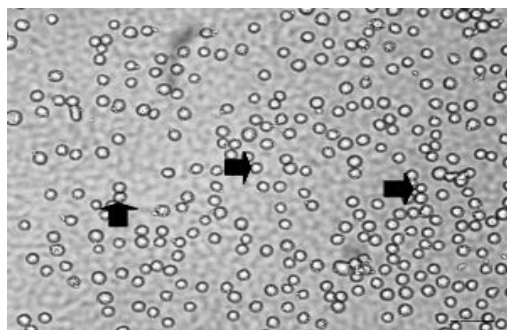
### Statistical Analysis

The different parameters measured from the normal, hypoxic and treated experimental groups of B50 neuronal cells were compared using mean and standard deviation (SD). The parameters were assayed in triplicate and repeated twice ( $n = 6$ ) and the results presented as the mean  $\pm$  SD. The Students't-test was used for testing the level of significance between two groups and a P-value less than 0.05 was considered to be significant using Microsoft Excel® package. For multiple treatment data, One-Way Analysis of Variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the least significant difference (LSD) between the groups.

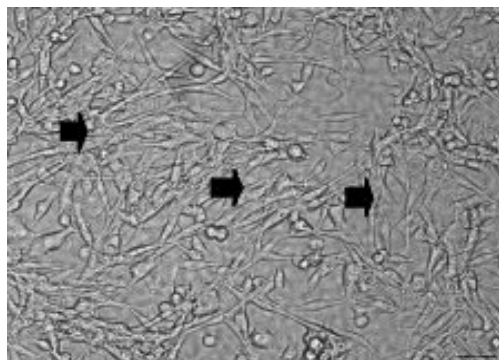
## RESULTS

### Morphological Studies

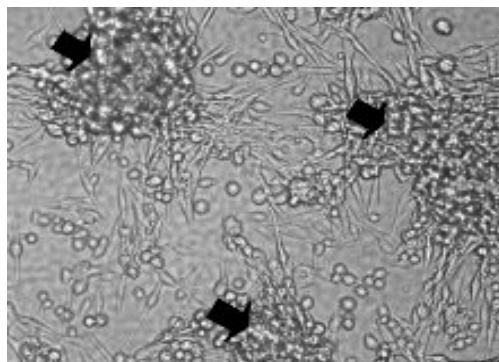
Morphological changes were observed in B50 cells cultured under hypoxia when compared to cells cultured in normoxia. The B50 cells in hypoxia showed clustered groups of neuronal B50 cells, evidence of degenerating, dying cells and already degenerated and dead neuronal B50 cells. The normal B50 neuronal cells cultured under normal incubator showed normal neuronal morphology (Figs. 1 and 2), when compared to B50 cells in hypoxia (Figs. 3 and 4).



**Fig. 1.** Representative of B50 cells (arrow) at the point of starting the culture at 21% O<sub>2</sub> and 5% CO<sub>2</sub>. B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS1 microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x 40 magnification



**Fig. 2.** Representative of B50 cells at 48 hrs of normal culture (21% O<sub>2</sub> and 5% CO<sub>2</sub>) and B50 cells (arrow). B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with IBM Image Solutions®. Scale bar = 5mm x 40 magnification



**Fig. 3.** Representative of hypoxic B50 cells at 48 hrs of culture (5% O<sub>2</sub> and 5% CO<sub>2</sub>) without drug administration, showing groups of degenerating cells (arrow). Groups of degenerating cells were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x 40 magnification

### *The Effect of Hypoxia on the Expression of Mu Opioid Receptor Gene in B50 cells*

The result of Mu receptor gene expression in B50 neuronal cells in normal, hypoxic and treated cells was studied using reverse transcription polymerase chain reaction (RT-PCR). The results showed that the RT-PCR experiments with the B50 neuronal cells in normal, hypoxic and treated cultures demonstrated positive gene expression of the Mu opioid receptors. The mRNA levels of Mu opioid receptors in hypoxic



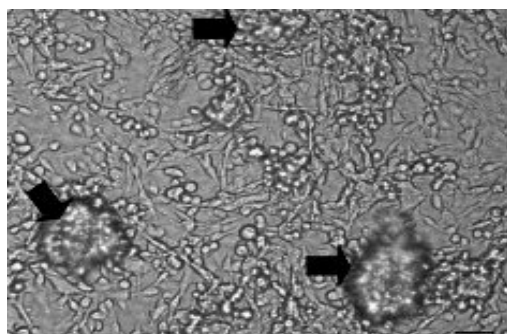


Fig. 4. Representative of B50 cells in hypoxia at 96 hrs of culture (5% O<sub>2</sub> and 5% CO<sub>2</sub>) with groups of degenerating cells (arrow). B50 cells was observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x 20 magnification

culture of B50 cells were expressed relative to Mu opioid receptors in B50 cells cultured under normal conditions, and these were also compared with mRNA levels of mu opioid receptors in hypoxic B50 cells treated with different receptor agonists (Fig. 5). The results showed that there were no significant difference in the levels of mu opioid receptor gene and mRNA expression between the normal, hypoxic and agonist treated cultured B50 cells.

#### Semi-quantitative RT-PCR of Cannabinoid CB<sub>1</sub> and MOR

MOR expression products were subjected to semi quantitative analysis using digital densitometric measurements. The decrease in the density of MOR RT-PCR products was not significant in untreated (MOR) hypoxic cells (93%)

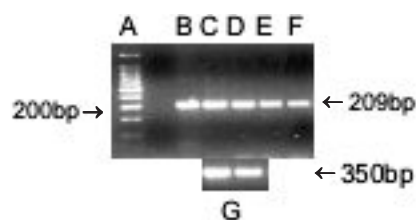


Fig. 5. The effect of ipioid treatment on MOR expression in cultured B50 cells in hypoxia A = DNA Ladder, B = Normal cells; C = Hypoxic cells; DEF = Hypoxic MOR treated G = Alpha Actin

and those treated with 10μM DAMGO (98%); 50μM DAMGO and 100μM DAMGO (98%). There was no significant change in densitometric measurements of mRNA area, mRNA volume, height and width respectively when compared with the normal control B50 cells (Table 1).

When the density of the RT-PCR products was normalised to alpha actin (100%), there was no observed difference in the level of the receptor mRNA expression of MOR in normal, hypoxic and treated cultured B50 cells. The results showed no significant difference between the normal MOR (119%); 10μM DAMGO (118%); 50μM DAMGO (111%) and 100μM DAMGO (118%).

Mu opioid receptor (MOR) expression in B50 neuronal cells in culture of which the total RNA was extracted from cultured B50 cells in normal (21%O<sub>2</sub>, 5%CO<sub>2</sub>), hypoxic (5%O<sub>2</sub>, 5%CO<sub>2</sub>) and hypoxia treated with MOR agonists. RT-PCR amplification was performed with specific MOR primers. The gel was electrophoresed and stained with ethidium bromide to visualise the intensity of the cDNA for MOR (209bp) and was normalised relative to alpha actin (350bp).

Table 1: Semi-quantitative RT-PCR product of MOR receptors in B50 cells in culture

Treatment type	Vol.mm <sup>3</sup>	Areamm <sup>2</sup>	STD ±	Density	Width	Height	% of Normal	% of Control
MOR Normal	11.20	4.04	0.32	621.12	2.78	1.56	100	119.57
MORHypoxia	04.55	1.94	0.24	583.40	0.76	2.54	93.93	112.31
10μM DAMGO	14.57	5.90	0.35	613.07	2.73	2.16	98.70	118.02
50μM DAMGO	12.48	5.34	0.34	579.21	1.59	3.37	93.27	111.50
100μM DAMGO	10.61	4.29	0.35	613.22	1.78	2.41	98.73	118.05
DAMGO/CTAP	11.29	4.44	0.33	631.09	2.79	1.59	101.61	121.49
Alpha actin	10.35	3.22	0.32	519.46	2.02	3.12	83.63	100

MOR receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal (21%O<sub>2</sub>, 5%CO<sub>2</sub>), hypoxic (5%O<sub>2</sub>, 5%CO<sub>2</sub>) and hypoxia treated with MOR agonists. The RT-PCR was amplified with specific MOR primers and gel was electrophoresed and stained with ethidium bromide and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control. (Data presented as mean ±SD; Student's t-test)

## DISCUSSION

The use of experimental approaches which include morphological assessments, cell live and dead trypan staining, viability and proliferation assay was used to assess neuronal injury more directly from hypoxia. Reactive oxygen species generated during hypoxia-ischaemia contribute to the cellular injury. Oxygen free-radicals serve as important signalling molecules that trigger cell death and apoptosis in neuronal cells (Durukan and Tatlisumak 2007; Lee et al. 2012). The findings of Mahura (2003), have shown that any neuroprotective agent against hypoxia and ischaemia should be able to reduce the generation of oxygen free radicals and limit  $\text{Ca}^{2+}$  influx into neuronal cerebral cells, which will invariably reduce inflammation and cell death.

This study has demonstrated that stimulation of opioid receptors proffers some protection by reducing neuronal cell injuries and deaths after treatments in hypoxic conditions but the benefit is reduced substantially with prolonged exposure durations and higher concentrations of the drugs, results not shown here. This supports the finding that the longer the neurons stayed in the culture media in the experimental hypoxic groups, the greater the extent of the neuronal cell injuries and death (Francis and Wei 2010; Du et al. 2012; Prentice et al. 2012). A possible explanation for this phenomenon is that prolonged hypoxia may cause a significant release and accumulation of endogenous glutamate which causes glutamate-induced toxicity and hence death of the cells (Nyakas et al. 1996; Yeh et al. 2008; Francis and Wei 2010).

It has been shown that in response to short-term hypoxia, the level of enkephalins, the endogenous agonists for opioid receptors sharply increases (Zhang et al. 2002; Khasabova et al. 2002; Kirbach and Golenhofen 2011). This showed that cortical neurons may release opioids during normal function and in response to hypoxic stress as a mechanism of self-protection against injury (Ma et al. 2005; Wallace et al. 2006; Lee et al. 2012). Zhang et al. (2002) showed that because of high levels of endogenous opioids which may already be present in the culture media after prolonged exposure to hypoxia, adding more agonist may not increase the protection by the drugs. However, cellular injury was observed in cultured B50 neurons in

hypoxic conditions which suggest that maturational differences exist between the different age group of the neurons. This supports the work of Zhang et al. (2000), which showed that opioid receptor expression increases significantly with development in both brain and cultured neurons. This is because the opioid receptor density increases with increase in the age of the neurons though the result from this present study shows that there was no difference in the  $\mu$  opioid receptor gene expression and no significant change in the expression densities of the  $\mu$  opioid receptors was observed. The more mature neurons may have greater dependence on this pathway to maintain neuronal function and therefore may be more susceptible to neuronal injury with opioid receptor inhibition (Xue et al. 2011; Lee et al. 2012). This observation suggests that the increase in opioid receptor agonist may compete with opioid receptor antagonist in terms of opioid receptor binding and thus reduce neuronal injury induced by opioid receptor inhibition during prolonged hypoxia (Wallace et al. 2006; Yu et al. 2009; Xue et al. 2011). This is in support of the findings in this study in which at higher concentration of the agonists and antagonist treatment against hypoxia could have led to complete inhibition of the agonists activities in cultured B50 cells in hypoxia.

Another issue is whether the differences in the expression levels of the various opioid receptors accounted for the observed phenomenon in cortical neurons. Past studies have demonstrated that  $\mu$ -opioid receptors are present at similar or even higher densities than  $\delta$ -opioid receptors in mammalian cortex, although  $\kappa$ -opioid receptor density is slightly lower (Zhang et al. 2006). This implies that the relative distribution and expression levels of opioid receptor subtypes within the cortex as a whole may not be a key factor in the observations shown in this work. This is because the results presented in this study showed that there were no observed differences in the receptor expression densities of  $\mu$ -opioid receptors in normoxic, hypoxic and hypoxic treated B50 cells.

It has been shown that intracellular  $\text{Ca}^{2+}$  levels were elevated during hypoxic exposure leading to irreversible cell injury while the inhibition of  $\text{Ca}^{2+}$  currents by opioid receptor stimulation by DAMGO, DSLET and ICI-199441, may serve as a neuroprotective mechanism in preventing  $\text{Ca}^{2+}$  overload (Andersen

2004; Bossy-Wetzel et al. 2004; Shimamura et al. 2012), and this could be the situation with the protection proffered by the  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid agonists used in this study. This mechanism of cellular regulation may be utilised during normal cell functioning and in response to oxidative stress like hypoxia (Andersen 2004; Shimamura et al. 2012). The  $\delta$ -,  $\mu$ - and  $\kappa$ -opioid receptors have many similarities such as seven transmembrane domains existing as 60% identical sequences and being coupled to  $G_{i/o}$  proteins. Connor and Christie (1999) had proposed that because of the common features of opioid receptors, the selectivity of these receptors for eliciting specific pathways does not lie in the differences between each opioid receptor subtype but in their association with other divergent types of G proteins. It was observed that each opioid receptor subtype also preferentially couples to specific G proteins apart from the  $G_{i/o}$  proteins that they generally couple (Zhang et al. 2002; Vermehren-Schmaedick et al. 2012). Examples of this preferential coupling include as seen in  $\delta$ -opioid receptors which are more efficiently coupled to  $G\alpha_{16}$  protein than either  $\mu$ - or  $\kappa$ -opioid receptors (Lee et al. 1998, Lee et al. 2012). Also  $\mu$ -receptor agonist (DAMGO) has been reported to have a selective coupling to  $G\alpha_{i1}$  and  $G\alpha_{oA}$  opioid receptors than other opioid agonists (Saidak et al. 2006). This shows that the opioid receptor agonists have selective activation of G-proteins in response to opioid receptor activation. This preferential coupling to other G protein subtypes may be the reason for the observed differences in the effect of the opioid agonists on the B50 cells treated in hypoxia.

### CONCLUSION

The results from the present study showed that hypoxia induced morphological changes in B50 cells in hypoxia while the Mu RT-PCR mRNA levels showed no appreciable changes in normal, hypoxic and cells treated with Mu opioid receptor agonists. The results show that B50 neuronal cells are susceptible to injurious effects of oxidative stress agents like hypoxia, as are most brain cells and the Mu opioid receptor agonist treatments showed that there were no changes in the level of Mu opioid receptor gene expression due to hypoxia or agonist treatment in cultured cortical neuronal B50 cells.

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